



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/70, 48/00, C07H 21/00, C12N 5/08		A1	(11) International Publication Number: WO 99/02167
			(43) International Publication Date: 21 January 1999 (21.01.99)
(21) International Application Number: PCT/US98/13961			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(22) International Filing Date: 6 July 1998 (06.07.98)			
(30) Priority Data: 08/888,982 7 July 1997 (07.07.97) US			
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 08/888,982 (CON) Filed on 7 July 1997 (07.07.97)			
(71) Applicant (for all designated States except US): ISIS PHARMACEUTICALS, INC. [US/US]; Carlsbad Research Center, 2292 Faraday Avenue, Carlsbad, CA 92008 (US).			
(72) Inventor; and (75) Inventor/Applicant (for US only): MONIA, Brett, P. [US/US]; 7605 Nueva Castilla Way, La Costa, CA 92009 (US).			
(74) Agents: LICATA, Jane, Massey et al.; Law Offices of Jane Massey Licata, 66 E. Main Street, Marlton, NJ 08053 (US).			
(54) Title: ANTISENSE OLIGONUCLEOTIDE MODULATION OF raf GENE EXPRESSION			
(57) Abstract Oligonucleotides are provided which are targeted to nucleic acids encoding human raf and capable of inhibiting raf expression. Methods of inhibiting the expression of human raf using oligonucleotides of the invention are also provided. The present invention further comprises methods of preventing or inhibiting hyperproliferation of cells and methods of treating abnormal proliferative conditions which employ oligonucleotides of the invention.			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Larvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

- 1 -

ANTISENSE OLIGONUCLEOTIDE MODULATION OF raf GENE EXPRESSION**INTRODUCTION**

This application is a continuation-in-part of United States Patent application Serial Number 08/756,806, filed 5 November 26, 1996, which is a U.S. National phase equivalent of PCT/US95/07111 filed May 31, 1995, which in turn is a foreign filing and continuation-in part of Serial Number 08/250,856 filed May 31, 1994. Each of these applications is assigned to the assignee of the present invention and is 10 incorporated herein in its entirety.

FIELD OF THE INVENTION

This invention relates to compositions and methods for modulating expression of raf, a naturally present cellular protein which has been implicated in abnormal cell 15 proliferation and tumor formation. Compositions and methods for modulating B-raf are provided. This invention is also directed to methods for inhibiting hyperproliferation of cells; these methods can be used diagnostically or therapeutically. Furthermore, this invention is directed to 20 treatment of conditions associated with expression of the raf gene.

- 2 -

BACKGROUND OF THE INVENTION

Alterations in the cellular genes which directly or indirectly control cell growth and differentiation are considered to be the main cause of cancer. The raf gene family includes three highly conserved genes termed A-, B- and c-raf (also called raf-1). Raf genes encode protein kinases that are thought to play important regulatory roles in signal transduction processes that regulate cell proliferation. Expression of the c-raf protein is believed to play a role in abnormal cell proliferation since it has been reported that 60% of all lung carcinoma cell lines express unusually high levels of c-raf mRNA and protein. Rapp et al., *The Oncogene Handbook*, E.P. Reddy, A.M Skalka and T. Curran, eds., Elsevier Science Publishers, New York, 1988, pp. 213-253. B-raf is strongly activated by oncogenic ras. Marais et al., (1997) *J. Biol. Chem.* 272: 4378-4383.

Oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. For example, workers in the field have now identified antisense, triplex and other oligonucleotide compositions which are capable of modulating expression of genes implicated in viral, fungal and metabolic diseases.

Antisense oligonucleotides have been safely administered to humans and clinical trials of several antisense oligonucleotide drugs, targeted both to viral and cellular gene products, are presently underway. The phosphorothioate oligonucleotide, ISIS 2922, has been shown to be effective against cytomegalovirus retinitis in AIDS patients. *BioWorld Today*, April 29, 1994, p 3. The oligonucleotide drug, ISIS5132/CGP69846A, a phosphorothioate deoxyoligonucleotide targeted to human c-raf, is currently in Phase I clinical trials. This compound has shown potent antitumor activity in a variety of animal models of human tumors. It is thus established that oligonucleotides in general, and those targeted to raf in particular, can be

- 3 -

useful therapeutic instrumentalities and can be useful in treatment of cells and animal subjects, especially humans.

Antisense oligonucleotide inhibition of gene expression has proven to be a useful tool in understanding the roles of raf gene products. An antisense oligonucleotide complementary to the first six codons of human c-raf has been used to demonstrate that the mitogenic response of T cells to interleukin-2 (IL-2) requires c-raf. Cells treated with the oligonucleotide showed a near-total loss of c-raf protein and a substantial reduction in proliferative response to IL-2. Riedel et al., *Eur. J. Immunol.* **1993**, 23, 3146-3150. Rapp et al. have disclosed expression vectors containing a raf gene in an antisense orientation downstream of a promoter, and methods of inhibiting raf expression by expressing an antisense Raf gene or a mutated Raf gene in a cell. WO application 93/04170. An antisense oligodeoxyribonucleotide complementary to codons 1-6 of murine c-Raf has been used to abolish insulin stimulation of DNA synthesis in the rat hepatoma cell line H4IIE. Tornkvist et al., *J. Biol. Chem.* **1994**, 269, 13919-13921. WO Application 93/06248 discloses methods for identifying an individual at increased risk of developing cancer and for determining a prognosis and proper treatment of patients afflicted with cancer comprising amplifying a region of the c-raf gene and analyzing it for evidence of mutation.

Denner et al. disclose antisense polynucleotides hybridizing to the gene for raf, and processes using them. WO 94/15645. Oligonucleotides hybridizing to human and rat raf sequences are disclosed.

Iversen et al. disclose heterotypic antisense oligonucleotides complementary to raf which are able to kill ras-activated cancer cells, and methods of killing raf-activated cancer cells. Numerous oligonucleotide sequences are disclosed, none of which are actually antisense oligonucleotide sequences. WO 94/23755.

- 4 -

SUMMARY OF THE INVENTION

The present invention provides oligonucleotides which are targeted to nucleic acids encoding human raf, particularly B-raf, and which are capable of inhibiting raf expression. 5 The oligonucleotides of the invention are believed to be useful both diagnostically and therapeutically, and are believed to be particularly useful in the methods of the present invention.

The present invention also comprises methods of 10 inhibiting the expression of human raf. These methods are believed to be useful both therapeutically and diagnostically as a consequence of the association between raf expression and hyperproliferation. These methods are also useful as tools, for example for detecting and determining the role of raf 15 expression in various cell functions and physiological processes and conditions and for diagnosing conditions associated with raf expression.

The present invention also comprises methods of inhibiting hyperproliferation of cells using oligonucleotides 20 of the invention. These methods are believed to be useful, for example in diagnosing raf-associated cell hyperproliferation. Methods of treating abnormal proliferative conditions are also provided. These methods employ the oligonucleotides of the invention. These methods are believed 25 to be useful both therapeutically and as clinical research and diagnostic tools.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a set of bar graphs showing initial screens of oligonucleotides targeted to human B-raf. Figure 1A shows 30 results for oligonucleotides ISIS 13720-13744. mRNA levels for both lower transcript (solid gray bars) and upper transcript (speckled bars) are shown. Figure 1B shows results for oligonucleotides 14132-14144, along with 13741, 13742 and

- 5 -

13743. mRNA levels for both upper transcript (solid gray bars) and lower transcript (speckled bars) are shown.

Figure 2 is a set of line graphs showing dose-response curves for reduction of B-raf mRNA levels in human A549 tumor cells by phosphorothioate oligodeoxynucleotides 13741 (open squares), 14144 (diamonds), 14529 (circles), 14530 (triangles) and 14531 (crosshatched squares) after a 4-hour treatment in the presence of lipofectin. Results are normalized to G3PDH and expressed as a percent of control. Figure 2A shows a dose-response curve for inhibition of the upper B-raf transcript. Figure 2B shows a dose-response curve for inhibition of the lower B-raf transcript.

Figure 3 is a set of line graphs showing dose-response curves for reduction of B-raf mRNA levels in human T24 tumor cells by phosphorothioate oligodeoxynucleotides 13741 (open squares), 14144 (diamonds), 14529 (circles), 14530 (triangles) and 14531 (crosshatched squares) after a 4-hour treatment in the presence of lipofectin. Results are normalized to G3PDH and expressed as a percent of control. Figure 3A shows a dose-response curve for inhibition of the upper B-raf transcript. Figure 3B shows a dose-response curve for inhibition of the lower B-raf transcript.

Figure 4 is a set of line graphs showing dose-response curves for reduction of B-raf mRNA levels in human T24 tumor cells by phosphorothioate oligodeoxynucleotides 13741 (squares) and 2'-methoxyethoxy oligonucleotides 15341 (diamonds), 15342 (circles) and 15344 (triangles) after a 4-hour treatment in the presence of lipofectin. Results are normalized to G3PDH and expressed as a percent of control. Figure 4A shows a dose-response curve for inhibition of the upper B-raf transcript. Figure 4B shows a dose-response curve for inhibition of the lower B-raf transcript.

- 6 -

DETAILED DESCRIPTION OF THE INVENTION

Malignant tumors develop through a series of stepwise, progressive changes that lead to the loss of growth control characteristic of cancer cells, i.e., continuous unregulated proliferation, the ability to invade surrounding tissues, and the ability to metastasize to different organ sites. Carefully controlled *in vitro* studies have helped define the factors that characterize the growth of normal and neoplastic cells and have led to the identification of specific proteins that control cell growth and differentiation. The raf genes are members of a gene family which encode related proteins termed A-, B- and c-raf. Raf genes code for highly conserved serine-threonine-specific protein kinases which are known to bind to the ras oncogene. They are part of a signal transduction pathway believed to consist of receptor tyrosine kinases, p21 ras, Raf protein kinases, Mek1 (ERK activator or MAPKK) kinases and ERK (MAPK) kinases, which ultimately phosphorylate transcription factors. Signaling through this pathway can mediate differentiation, proliferation or oncogenic transformation in different cellular contexts. Marais et al., (1997) *J. Biol. Chem.* 272: 4378-4383. Thus, raf kinases are believed to play a fundamental role in the normal cellular signal transduction pathway, coupling a multitude of growth factors to their net effect, cellular proliferation. Because raf proteins are direct downstream effectors of ras protein function, therapies directed against raf kinases are believed to be useful in treatment of ras-dependent tumors. Monia et al. (1996) *Nature Med.* 2:668-675. The raf kinases are differentially regulated and expressed; c-raf, also known as raf-1, is the most thoroughly characterized and is expressed in all organs and in all cell lines that have been examined. A- and B-raf are highly expressed in urogenital and brain tissues, respectively. Because B-raf is highly expressed in neural tissues it was once thought to be limited to these tissues but it has since

- 7 -

been found to be more widely expressed. Although all the raf kinases are bound by ras following ras stimulation, B-raf is most strongly activated (phosphorylated) by oncogenic ras, and may be the primary target of oncogenic ras in cell transformation. Marais et al., (1997) *J. Biol. Chem.* 272: 4378-4383.

Certain abnormal proliferative conditions are believed to be associated with raf expression and are, therefore, believed to be responsive to inhibition of raf expression. Abnormally high levels of expression of the raf protein are also implicated in transformation and abnormal cell proliferation. These abnormal proliferative conditions are also believed to be responsive to inhibition of raf expression. Examples of abnormal proliferative conditions are hyperproliferative disorders such as cancers, tumors, hyperplasias, pulmonary fibrosis, angiogenesis, psoriasis, atherosclerosis and smooth muscle cell proliferation in the blood vessels, such as stenosis or restenosis following angioplasty. The cellular signalling pathway of which raf is a part has also been implicated in inflammatory disorders characterized by T-cell proliferation (T-cell activation and growth), such as tissue graft rejection, endotoxin shock, and glomerular nephritis, for example.

It has now been found that elimination or reduction of raf expression may halt or reverse abnormal cell proliferation. This has been found even when levels of raf expression are not abnormally high. There is a great desire to provide compositions of matter which can modulate the expression of raf. It is greatly desired to provide methods of detection of nucleic acids encoding raf in cells, tissues and animals. It is also desired to provide methods of diagnosis and treatment of abnormal proliferative conditions associated with abnormal raf expression. In addition, kits and reagents for detection and study of nucleic acids encoding raf are desired. "Abnormal" raf expression is defined herein

- 8 -

as abnormally high levels of expression of the raf protein, expression of an abnormal or mutant raf protein, or any level of raf expression in an abnormal proliferative condition or state.

5 The present invention employs oligonucleotides targeted to nucleic acids encoding raf. This relationship between an oligonucleotide and its complementary nucleic acid target to which it hybridizes is commonly referred to as "antisense". "Targeting" an oligonucleotide to a chosen nucleic acid
10 target, in the context of this invention, is a multistep process. The process usually begins with identifying a nucleic acid sequence whose function is to be modulated. This may be, as examples, a cellular gene (or mRNA made from the gene) whose expression is associated with a particular disease
15 state, or a foreign nucleic acid from an infectious agent. In the present invention, the target is a nucleic acid encoding raf; in other words, the raf gene or mRNA expressed from the raf gene. The targeting process also includes determination of a site or sites within the nucleic acid
20 sequence for the oligonucleotide interaction to occur such that the desired effect- modulation of gene expression- will result. Once the target site or sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well
25 and with sufficient specificity, to give the desired modulation.

 In the context of this invention "modulation" means either inhibition or stimulation. Inhibition of raf gene expression is presently the preferred form of modulation.
30 This modulation can be measured in ways which are routine in the art, for example by Northern blot assay of mRNA expression or Western blot assay of protein expression as taught in the examples of the instant application. Effects on cell proliferation or tumor cell growth can also be measured, as
35 taught in the examples of the instant application.

- 9 -

"Hybridization", in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between them. Adenine and thymine are examples of complementary bases which form two hydrogen bonds between them. "Specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment or, in the case of *in vitro* assays, under conditions in which the assays are conducted.

In preferred embodiments of this invention, oligonucleotides are provided which are targeted to mRNA encoding B-raf. In accordance with this invention, persons of ordinary skill in the art will understand that mRNA includes not only the coding region which carries the information to encode a protein using the three letter genetic code, including the translation start and stop codons, but also associated ribonucleotides which form a region known to such persons as the 5'-untranslated region, the 3'-untranslated region, the 5' cap region, intron regions and intron/exon or splice junction ribonucleotides. Thus,

- 10 -

oligonucleotides may be formulated in accordance with this invention which are targeted wholly or in part to these associated ribonucleotides as well as to the coding ribonucleotides. In preferred embodiments, the
5 oligonucleotide is targeted to a translation initiation site (AUG codon) or sequences in the coding region, 5' untranslated region or 3'-untranslated region of the human B-raf mRNA. The functions of messenger RNA to be interfered with include all vital functions such as translocation of the RNA to the site
10 for protein translation, actual translation of protein from the RNA, splicing or maturation of the RNA and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with the RNA function is to cause interference with raf protein expression.

15 The present invention provides oligonucleotides for modulation of raf gene expression. Such oligonucleotides are targeted to nucleic acids encoding raf. Oligonucleotides and methods for modulation of B-raf are provided; however, compositions and methods for modulating expression of other
20 forms of raf are also believed to have utility and are comprehended by this invention. As hereinbefore defined, "modulation" means either inhibition or stimulation. Inhibition of raf gene expression is presently the preferred form of modulation.

25 In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The term "oligonucleotide" also includes oligomers comprising
30 non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake and increased stability in the presence of nucleases.

- 11 -

Certain preferred oligonucleotides of this invention are chimeric oligonucleotides. "Chimeric oligonucleotides" or "chimeras", in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region of modified nucleotides that confers one or more beneficial properties (such as, for example, increased nuclease resistance, increased uptake into cells, increased binding affinity for the RNA target) and a region that is a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligos are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. In one preferred embodiment, a chimeric oligonucleotide comprises at least one region modified to increase target binding affinity, and, usually, a region that acts as a substrate for RNase H. Affinity of an oligonucleotide for its target (in this case a nucleic acid encoding raf) is routinely determined by measuring the T_m of an oligonucleotide/target pair, which is the temperature at which the oligonucleotide and target dissociate; dissociation is detected spectrophotometrically. The higher the T_m , the greater the affinity of the oligonucleotide for the target. In a more preferred embodiment, the region of the oligonucleotide which is modified to increase raf mRNA binding affinity comprises at least one nucleotide modified at the 2' position of the sugar,

- 12 -

most preferably a 2'-O-alkyl, 2'-O-alkyl-O-alkyl or 2'-fluoro-modified nucleotide. Such modifications are routinely incorporated into oligonucleotides and these oligonucleotides have been shown to have a higher T_m (i.e., higher target
5 binding affinity) than 2'-deoxyoligonucleotides against a given target. The effect of such increased affinity is to greatly enhance antisense oligonucleotide inhibition of raf gene expression. RNase H is a cellular endonuclease that cleaves the RNA strand of RNA:DNA duplexes; activation of this
10 enzyme therefore results in cleavage of the RNA target, and thus can greatly enhance the efficiency of antisense inhibition. Cleavage of the RNA target can be routinely demonstrated by gel electrophoresis. In another preferred embodiment, the chimeric oligonucleotide is also modified to
15 enhance nuclease resistance. Cells contain a variety of exo- and endo-nucleases which can degrade nucleic acids. A number of nucleotide and nucleoside modifications have been shown to make the oligonucleotide into which they are incorporated more resistant to nuclease digestion than the native
20 oligodeoxynucleotide. Nuclease resistance is routinely measured by incubating oligonucleotides with cellular extracts or isolated nuclease solutions and measuring the extent of intact oligonucleotide remaining over time, usually by gel electrophoresis. Oligonucleotides which have been modified to
25 enhance their nuclease resistance survive intact for a longer time than unmodified oligonucleotides. A variety of oligonucleotide modifications have been demonstrated to enhance or confer nuclease resistance. Oligonucleotides which contain at least one phosphorothioate modification are
30 presently more preferred. In some cases, oligonucleotide modifications which enhance target binding affinity are also, independently, able to enhance nuclease resistance. A discussion of antisense oligonucleotides and some desirable modifications can be found in De Mesmaeker et al., 1995, *Acc.*
35 *Chem. Res.* 28:366-374.

- 13 -

Specific examples of some preferred oligonucleotides envisioned for this invention include those containing modified backbones, for example, phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are oligonucleotides with phosphorothioate backbones and those with heteroatom backbones, particularly $\text{CH}_2\text{-NH-O-CH}_2$, $\text{CH}_2\text{-N(CH}_3\text{)-O-CH}_2$ [known as a methylene(methylimino) or MMI backbone], $\text{CH}_2\text{-O-N(CH}_3\text{)-CH}_2$, $\text{CH}_2\text{-N(CH}_3\text{)-N(CH}_3\text{)-CH}_2$ and $\text{O-N(CH}_3\text{)-CH}_2\text{-CH}_2$ backbones, wherein the native phosphodiester backbone is represented as O-P-O-CH_2). The amide backbones disclosed by De Mesmaeker et al. (1995, *Acc. Chem. Res.* 28:366-374) are also preferred. Also preferred are oligonucleotides having morpholino backbone structures (Summerton and Weller, U.S. Patent 5,034,506). In other preferred embodiments, such as the peptide nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide is replaced with a polyamide backbone, the nucleobases being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone (Nielsen et al., *Science*, 1991, 254, 1497). Oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH, SH, SCH_3 , F, OCN, OCH_2OCH_3 , $\text{OCH}_2\text{O(CH}_2\text{)}_n\text{CH}_3$, $\text{O(CH}_2\text{)}_n\text{NH}_2$ or $\text{O(CH}_2\text{)}_n\text{CH}_3$ where n is from 1 to about 10; C_1 to C_{10} lower alkyl, alkoxyalkoxy (also known in the art as O-alkyl-O-alkyl), substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF_3 ; OCF_3 ; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH_3 ; SO_2CH_3 ; ONO_2 ; NO_2 ; N_3 ; NH_2 ; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A preferred

- 14 -

modification includes 2'-methoxyethoxy [2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE] (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486). Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-propoxy (2'-OCH₂CH₂CH₃) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group.

Oligonucleotides may also include, additionally or alternatively, nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine, 5-me pyrimidines, particularly 5-methylcytosine (also referred to as 5-methyl-2'deoxycytosine and often referred to in the art as 5-me-C), 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentobiosyl HMC, as well as synthetic nucleobases, e.g., 2-aminoadenine, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N⁶(6-aminohexyl)adenine and 2,6-diaminopurine. Kornberg, A., *DNA Replication*, W.H. Freeman & Co., San Francisco, 1980, pp75-77; Gebeyehu, G., et al., 1987, *Nucl. Acids Res.* 15:4513). A "universal" base known in the art, e.g., inosine, may be included. 5-me-C substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., in Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide

- 15 -

one or more moieties or conjugates which enhance the activity or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, a cholesteryl moiety (Letsinger et al.,
5 *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765), a thiocholesterol (Oberhauser
10 et al., *Nucl. Acids Res.*, 1992, 20, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10, 111; Kabanov et al., *FEBS Lett.*, 1990, 259, 327; Svinarchuk et al., *Biochimie*, 1993, 75, 49), a phospholipid, a polyamine or a polyethylene glycol chain
15 (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651). Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides are known in the art, for example, U.S.
20 Patents No. 5,138,045, No. 5,218,105 and No. 5,459,255.

The oligonucleotides of the invention may be provided as prodrugs, which comprise one or more moieties which are cleaved off, generally in the body, to yield an active oligonucleotide. One example of a prodrug approach is
25 described by Imbach et al. in WO Publication 94/26764.

It is not necessary for all positions in a given oligonucleotide to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligonucleotide or even at within a
30 single nucleoside within an oligonucleotide. The present invention also includes chimeric oligonucleotides as hereinbefore defined.

The oligonucleotides in accordance with this invention preferably are from about 8 to about 50 nucleotides in length.
35 In the context of this invention it is understood that this

- 16 -

encompasses non-naturally occurring oligomers as hereinbefore described, having 8 to 50 monomers.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is also well known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives. It is also well known to use similar techniques and commercially available modified amidites and controlled-pore glass (CPG) products such as biotin, fluorescein, acridine or psoralen-modified amidites and/or CPG (available from Glen Research, Sterling VA) to synthesize fluorescently labeled, biotinylated or other modified oligonucleotides such as cholesterol-modified oligonucleotides.

It has now been found that certain oligonucleotides targeted to portions of the B-raf mRNA are useful for inhibiting raf expression. Inhibition of B-raf expression using antisense oligonucleotides is believed to be useful for interfering with cell hyperproliferation. In the methods of the invention, tissues or cells are contacted with oligonucleotides. In the context of this invention, to "contact" tissues or cells with an oligonucleotide or oligonucleotides means to add the oligonucleotide(s), usually in a liquid carrier, to a cell suspension or tissue sample, either in vitro or ex vivo, or to administer the oligonucleotide(s) to cells or tissues within an animal.

For therapeutics, methods of inhibiting hyperproliferation of cells and methods of preventing and treating abnormal proliferative conditions are provided. The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill in the art.

- 17 -

In general, for therapeutics, a patient suspected of needing such therapy is given an oligonucleotide in accordance with the invention, commonly in a pharmaceutically acceptable carrier, in amounts and for periods which will vary depending upon the nature of the particular disease, its severity and the patient's overall condition. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous drip or infusion, subcutaneous, intraperitoneal or intramuscular injection, pulmonary administration, e.g., by inhalation or insufflation, or intrathecal or intraventricular administration. For oral administration, it has been found that oligonucleotides with at least one 2'-substituted ribonucleotide are particularly useful because of their absorption and distribution characteristics. U.S. Patent 5,591,721 (Agrawal et al.). Oligonucleotides with at least one 2'-methoxyethyl modification are believed to be particularly useful for oral administration.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions for parenteral, intrathecal or intraventricular administration may include sterile aqueous

- 18 -

solutions which may also contain buffers, diluents and other suitable additives.

In addition to such pharmaceutical carriers, cationic lipids may be included in the formulation to facilitate oligonucleotide uptake. One such composition shown to facilitate uptake is Lipofectin (BRL, Bethesda MD).

Dosing is dependent on severity and responsiveness of the condition to be treated, with course of treatment lasting from several days to several months or until a cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be calculated based on IC₅₀'s or EC₅₀'s in *in vitro* and *in vivo* animal studies. For example, given the molecular weight of compound (derived from oligonucleotide sequence and chemical structure) and an effective dose such as an IC₅₀, for example (derived experimentally), a dose in mg/kg is routinely calculated.

The present invention is also suitable for diagnosing abnormal proliferative states in tissue or other samples from patients suspected of having a hyperproliferative disease such as cancer, psoriasis or blood vessel restenosis or atherosclerosis. The ability of the oligonucleotides of the present invention to inhibit cell proliferation may be employed to diagnose such states. A number of assays may be formulated employing the present invention, which assays will commonly comprise contacting a tissue sample with an oligonucleotide of the invention under conditions selected to permit detection and, usually, quantitation of such inhibition. Similarly, the present invention can be used to distinguish raf-associated, or, particularly, B-raf-associated

- 19 -

tumors from tumors having other etiologies, in order that an efficacious treatment regime can be designed.

The oligonucleotides of this invention may also be used for research purposes. Thus, the specific hybridization
5 exhibited by the oligonucleotides may be used for assays, purifications, cellular product preparations and in other methodologies which may be appreciated by persons of ordinary skill in the art.

The oligonucleotides of the invention are also useful
10 for detection and diagnosis of raf expression. For example, radiolabeled oligonucleotides can be prepared by ³²P labeling at the 5' end with polynucleotide kinase. Sambrook et al., *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989, Volume 2, p. 10.59. Radiolabeled
15 oligonucleotides are then contacted with tissue or cell samples suspected of raf expression and the sample is washed to remove unbound oligonucleotide. Radioactivity remaining in the sample indicates bound oligonucleotide (which in turn indicates the presence of raf) and can be quantitated using
20 a scintillation counter or other routine means. Radiolabeled oligo can also be used to perform autoradiography of tissues to determine the localization, distribution and quantitation of raf expression for research, diagnostic or therapeutic purposes. In such studies, tissue sections are treated with
25 radiolabeled oligonucleotide and washed as described above, then exposed to photographic emulsion according to routine autoradiography procedures. The emulsion, when developed, yields an image of silver grains over the regions expressing raf. Quantitation of the silver grains permits raf expression
30 to be detected.

Analogous assays for fluorescent detection of raf expression can be developed using oligonucleotides of the invention which are conjugated with fluorescein or other fluorescent tag instead of radiolabeling. Such conjugations
35 are routinely accomplished during solid phase synthesis using

- 20 -

fluorescently labeled amidites or CPG (e.g., fluorescein-labeled amidites and CPG available from Glen Research, Sterling VA. See 1993 *Catalog of Products for DNA Research*, Glen Research, Sterling VA, p. 21).

5 Each of these assay formats is known in the art. One of skill could easily adapt these known assays for detection of raf expression in accordance with the teachings of the invention providing a novel and useful means to detect raf expression.

10 **Oligonucleotide inhibition of B-raf expression**

 The oligonucleotides shown in Table 1 were designed using the Genbank B-raf sequence HUMBRAF (SEQ ID NO: 42; Genbank listings M95712;M95720;x54072), synthesized and tested for inhibition of B-raf mRNA expression in T24 bladder
15 carcinoma cells or A549 lung carcinoma cells using a Northern blot assay.

- 21 -

Table 1

Human B-raf Kinase Antisense Oligonucleotides

(All are phosphorothioate oligodeoxynucleotides)

	<u>Isis #</u>	<u>Sequence (5' - 3')</u>	<u>Site</u>	<u>SEO ID NO:</u>
5	13720	ATTTTGAAGGAGACGGACTG	coding	1
	13721	TGGATTTTGAAGGAGACGGA	coding	2
	13722	CGTTAGTTAGTGAGCCAGGT	coding	3
	13723	ATTTCTGTAAGGCTTTCACG	coding	4
	13724	CCCGTCTACCAAGTGTTTTC	coding	5
10	13725	AATCTCCCAATCATCACTCG	coding	6
	13726	TGCTGAGGTGTAGGTGCTGT	coding	7
	13727	TGTAAGTCTGAGGTGTAGG	coding	8
	13728	TGTCGTGTTTTCTGAGTAC	coding	9
	13729	AGTTGTGGCTTTGTGGAATA	coding	10
15	13730	ATGGAGATGGTGATACAAGC	coding	11
	13731	GGATGATTGACTTGCGTGT	coding	12
	13732	AGGTCTCTGTGGATGATTGA	coding	13
	13733	ATTCTGATGACTTCTGGTGC	coding	14
	13734	GCTGTATGGATTTTATCTT	coding	15
20	13735	TACAGAACAATCCCAAATGC	coding	16
	13736	ATCCTCGTCCCACCATAAAA	coding	17
	13737	CTCTCATCTCTTTCTTTTT	coding	18
	13738	GTCTCTCATCTCTTTCTTT	coding	19
	13739	CCGATTCAAGGAGGTCTG	coding	20
25	13740	TGGATGGGTGTTTTTGAGA	coding	21
	13741	CTGCCTGGATGGGTGTTTTT	coding	22
	14144	GGACAGGAAACGCACCATAT	coding	23
	14143	CTCATTTGTTTCAGTGGACA	stop codon	24

- 22 -

	<u>Isis #</u>	<u>Sequence (5' - 3')</u>	<u>Site</u>	<u>SEQ ID NO:</u>
	14142	TCTCTCACTCATTTGTTTCA	stop codon	25
	14141	ACTCTCTCACTCATTTGTTT	stop codon	26
	14140	GAACTCTCTCACTCATTTGT	coding	27
	14139	TCCTGAACTCTCTCACTCAT	coding	28
5	14138	TTGCTACTCTCCTGAACTCT	3' UTR	29
	14137	TTTGTTGCTACTCTCCTGAG	coding	30
	14136	CTTTTGTTGCTACTCTCCTG	3' UTR	31
	13742	GCTACTCTCCTGAACTCTCT	3' UTR	32
	14135	TTCCTTTTGTTGCTACTCTC	3' UTR	33
10	14134	ATTTATTTTCCTTTTGTTGC	coding	34
	14133	ATATGTTCAATTTATTTTCCT	coding	35
	13743	TTTATTTTCCTTTTGTTGCT	3' UTR	36
	13744	TGTTCAATTTATTTTCCTTTT	coding	37
	14132	ATTTAACATATAAGCAAACA	coding	38
15	14529	CTGCCTGGTACCCTGTTTTT	5 mismatch	39
	14530	CTGCCTGGAAGGGTGTTTTT	1 mismatch	40
	14531	CTGCCTGGTACGGTGTTTTT	3 mismatch	41

There are multiple B-raf transcripts. The two most prevalent transcripts were quantitated after oligonucleotide treatment.

20 These transcripts run at approximately 8.5 kb (upper transcript) and 4.7 kb (lower transcript) under the gel conditions used. Both transcripts are translated into B-raf protein in cells. In the initial screen, A549 cells were treated with oligonucleotides at a concentration of 200 nM

25 oligonucleotide for four hours in the presence of lipofectin. Results were normalized and expressed as a percent of control. A graph showing oligonucleotide effect on levels of B-raf mRNA (both upper and lower transcripts) is shown in Figure 1 (panels A and B). In this initial screen, oligonucleotides

30 giving a reduction of either B-raf mRNA transcript of

- 23 -

approximately 30% or greater were considered active. According to this criterion, oligonucleotides 13722, 13724, 13726, 13727, 13728, 13730, 13732, 13733, 13736, 13739, 13740, 13741, 13742, 13743, 14135, 14136, 14138 and 14144 were found
5 to be active. These sequences (SEQ ID NO: 3, 5, 7, 8, 9, 11, 13, 14, 17, 20, 21, 22, 32, 36, 33, 31, 29 and 23, respectively) are therefore preferred. Of these, oligonucleotides 13727, 13730, 13740, 13741, 13743 and 14144 (SEQ ID NO: 8, 11, 21, 22, 36 and 23, respectively) showed 40-
10 50% inhibition of one or both B-raf transcripts in at least one assay. These sequences are therefore more preferred. In one of the two assays, ISIS 14144 (SEQ ID NO: 23) reduced levels of both transcripts by 50-60% and ISIS 13741 (SEQ ID NO: 22) reduced both transcripts by 65-70%. These two
15 sequences are therefore highly preferred.

Dose response experiments were done in both T24 cells and A549 cells for the two most active oligonucleotides, ISIS 13741 and ISIS 14144 (SEQ ID NO: 22 and 23), along with mismatch control sequences having 1, 3 or 5 mismatches of the
20 ISIS 13741 sequence (SEQ ID NO: 22). Figure 2 shows dose-response curves for reduction of B-raf mRNA levels in A549 cells by these oligonucleotides (all are phosphorothioate oligodeoxynucleotides) after a 4-hour treatment in the presence of lipofectin. Results are normalized to G3PDH and
25 expressed as a percent of control. Figure 2A shows a dose-response curve for inhibition of the upper B-raf transcript. ISIS 13741 and 14144 had almost identical activity in this assay, with IC50s between 250 and 300 nM. The mismatch controls had no activity (ISIS 14531) or slight activity, with
30 a maximum inhibition of less than 20% at the 400 nM dose (ISIS 14530, ISIS 14529). Figure 2B shows a dose-response curve for inhibition of the lower B-raf transcript in A549 cells. Against the lower transcript, ISIS 13741 and ISIS 14144 had IC50s of approximately 350 and 275 nM, respectively in this
35 assay, with the mismatch controls never achieving 50%

- 24 -

inhibition at concentrations up to 400 nM. Therefore, ISIS 13741 and 14144 are preferred.

Figure 3 shows dose-response curves for reduction of B-raf mRNA levels in T24 cells by these oligonucleotides (all 5 are phosphorothioate oligodeoxynucleotides) after a 4-hour treatment in the presence of lipofectin. Results are normalized to G3PDH and expressed as a percent of control. Figure 3A shows a dose-response curve for inhibition of the upper B-raf transcript. ISIS 13741 and 14144 were again most 10 active, with IC50s of approximately 100 nM and 275 nM, respectively, in this assay. The mismatch controls 14529 and 14531 had no activity, and the mismatch control 14530 achieved a maximum reduction of raf mRNA of approximately 20% at a 400 nM dose. Figure 3B shows a dose-response curve for inhibition 15 of the lower B-raf transcript in T24 cells. Against the lower transcript, ISIS 13741 had an IC50 of approximately 100-125 nM and ISIS 14144 had an IC50 of approximately 250 nM in this assay, with the mismatch controls completely inactive. Therefore ISIS 13741 and 14144 are preferred.

20 2'-Methoxyethoxy (2'-MOE) oligonucleotides targeted to B-raf:

The oligonucleotides shown in Table 2 were synthesized. Nucleotides shown in **bold** are 2'-MOE. 2'-MOE cytosines are all 5-methylcytosines. For backbone linkage, "s" indicates phosphorothioate (P=S) and "o" indicates phosphodiester (P=O).

25

Table 2

2'-MOE oligonucleotides targeted to human B-raf

(bold=2'-MOE)		
<u>ISIS#</u>	<u>Sequence/modification</u>	<u>SEQ ID NO:</u>
13741	CsTsGsCsCsTsGsGsAsTsGsGsGsTsGsTsTsTsTsT	22
30 15339	CsTsGsCsCsTsGsGsAsTsGsGsGsTsGsTsTsTsTsT	22
15340	CoToGoCoCoToGoGoAoToGsGsGsTsGsTsTsTsTsT	22
15341	CsTsGsCsCsTsGsGsAsTsGsGsGsTsGsTsTsTsTsT	22

- 25 -

15342	CoToGoCoCsTsGsGsAsTsGsGsGsTsGoToToToToT	22
15343	CsTsGsCsCsTsGsGsAsToGoGoGoToGoToToToToT	22
15344	CsTsGsCsCsTsGsGsAsTsGsGsGsTsGsTsTsTsTsT	22

These oligonucleotides were tested for their ability to reduce
 5 B-raf mRNA levels in T24 cells. Dose response curves for ISIS
 13741, 15341, 15342 and 15344 are shown in Figure 4A. Figure
 4A shows the effect of these oligonucleotides on the lower B-
 raf transcript and Figure 4B shows the effect on the upper
 transcript. Against the lower transcript, ISIS 13741 (P=S
 10 deoxy) and ISIS 15344 (P=S deoxy/MOE) had IC50s of
 approximately 250 nM. The other two compounds tested, ISIS
 15341 and 15342, did not achieve 50% inhibition at doses up
 to 400 nM. Against the upper transcript, ISIS 13741 and 15344
 demonstrated IC50s of approximately 150 nM, ISIS 15341
 15 demonstrated an IC50 of approximately 200 nM and ISIS 15342
 did not achieve 50% reduction at doses up to 400 nM. Based
 on these results, ISIS 15341, 13741 and 15344 are preferred.
 The following examples are provided for illustrative purposes
 only and are not intended to limit the invention.

20 EXAMPLES

Example 1 Synthesis and Characterization of Oligonucleotides

Unmodified oligodeoxynucleotides are synthesized on an
 automated DNA synthesizer (Applied Biosystems model 380B)
 25 using standard phosphoramidite chemistry with oxidation by
 iodine. β -cyanoethyl-diisopropyl-phosphoramidites are
 purchased from Applied Biosystems (Foster City, CA). For
 phosphorothioate oligonucleotides, the standard oxidation
 bottle was replaced by a 0.2 M solution of ^3H -1,2-
 30 benzodithiole-3-one 1,1-dioxide in acetonitrile for the
 stepwise thiation of the phosphite linkages. The thiation

- 26 -

cycle wait step was increased to 68 seconds and was followed by the capping step.

2'-methoxy oligonucleotides were synthesized using 2'-methoxy β -cyanoethyl-diisopropyl-phosphoramidites (Chemgenes, Needham MA) and the standard cycle for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds. Other 2'-alkoxy oligonucleotides were synthesized by a modification of this method, using appropriate 2'-modified amidites such as those available from Glen Research, Inc., Sterling, VA.

2'-fluoro oligonucleotides were synthesized as described in Kawasaki et al., *J. Med. Chem.* **1993**, 36, 831-841. Briefly, the protected nucleoside N⁶-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9- β -D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'- α -fluoro atom is introduced by a S_N2-displacement of a 2'- β -O-triflyl group. Thus N⁶-benzoyl-9- β -D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N⁶-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl- (DMT) and 5'-DMT-3'-phosphoramidite intermediates.

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9- β -D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

- 27 -

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2, 2'-anhydro-1- β -D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to
5 obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N⁴-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-
10 3'phosphoramidites.

2'-(2-methoxyethyl)-modified amidites are synthesized according to Martin, P., *Helv. Chim. Acta* **1995**, 78,486-504. For ease of synthesis, the last nucleotide was a deoxynucleotide. 2'-O-CH₂CH₂OCH₃-cytosines may be 5-methyl
15 cytosines.

Synthesis of 5-Methyl cytosine monomers:

2,2'-Anhydro[1-(β -D-arabinofuranosyl)-5-methyluridine]:

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenyl-
20 carbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced
25 pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted
30 and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid which was crushed to a light tan powder (57 g, 85% crude yield). The material was used as is for further reactions.

- 28 -

2'-O-Methoxyethyl-5-methyluridine:

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH₃CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH₂Cl₂/acetone/MeOH (20:5:3) containing 0.5% Et₃NH. The residue was dissolved in CH₂Cl₂ (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH₃CN (200 mL). The residue was dissolved in CHCl₃ (1.5 L) and extracted with 2x500 mL of saturated NaHCO₃ and 2x500 mL of saturated NaCl. The organic phase was dried over Na₂SO₄, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/-Hexane/Acetone (5:5:1) containing 0.5% Et₃NH. The pure

- 29 -

fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

5 3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at
10 room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in CHCl₃ (800 mL) and extracted with
15 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl₃. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel
20 column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine:

A first solution was prepared by dissolving 3'-O-acetyl-2'-
25 O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH₃CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH₃CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl₃ was added dropwise, over a 30 minute
30 period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the later solution. The resulting reaction mixture was

- 30 -

stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO₃ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH₄OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH₃ gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N⁴-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl₃ (700 mL) and extracted with saturated NaHCO₃ (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO₄ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane

- 31 -

(1:1) containing 0.5% Et₃NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N⁴-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite:

N⁴-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH₂Cl₂ (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO₃ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH₂Cl₂ (300 mL), and the extracts were combined, dried over MgSO₄ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc\Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

5-methyl-2'-deoxycytidine (5-me-C) containing oligonucleotides were synthesized according to published methods (Sanghvi et al., 1993, *Nucl. Acids Res.* 21:3197-3203) using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

Oligonucleotides having methylene(methylimino) backbones are synthesized according to U.S. Patent 5,378,825, which is coassigned to the assignee of the present invention and is incorporated herein in its entirety. Other nitrogen-containing backbones are synthesized according to WO 92/20823 which is also coassigned to the assignee of the present invention and incorporated herein in its entirety.

Oligonucleotides having amide backbones are synthesized according to De Mesmaeker et al., *Acc. Chem. Res.* 1995, 28, 366-374. The amide moiety is readily accessible by simple and

- 32 -

well known synthetic methods and is compatible with the conditions required for solid phase synthesis of oligonucleotides.

Oligonucleotides with morpholino backbones are synthesized according to U.S. Patent 5,034,506 (Summerton and Weller).
5 Peptide-nucleic acid (PNA) oligomers are synthesized according to P.E. Nielsen et al., *Science* **1991**, 254, 1497).

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium
10 hydroxide at 55°C for 18 hours, the oligonucleotides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative
15 amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ³¹P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., 1991, *J. Biol. Chem.*, 266:18162-18171. Results
20 obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 2 Northern blot analysis of inhibition of B-raf mRNA expression

The human urinary bladder cancer cell line T24 and the
25 human lung tumor cell line A549 were obtained from the American Type Culture Collection (Rockville MD). T24 cells were grown in McCoy's 5A medium with L-glutamine and A549 cells were grown in DMEM low glucose medium (Gibco BRL, Gaithersburg MD), supplemented with 10% heat-inactivated fetal
30 calf serum and 50 U/ml each of penicillin and streptomycin. Cells were seeded on 100 mm plates. When they reached 70% confluency, they were treated with oligonucleotide. Plates were washed with 10 ml prewarmed PBS and 5 ml of Opti-MEM reduced-serum medium containing 2.5 µl DOTMA per 100 nM

- 33 -

oligonucleotide. Oligonucleotide with lipofectin was then added to the desired concentration. After 4 hours of treatment, the medium was replaced with appropriate medium (McCoy's or DMEM low glucose). Cells were harvested 24 to 72 5 hours after oligonucleotide treatment and RNA was isolated using a standard CsCl purification method. Kingston, R.E., in *Current Protocols in Molecular Biology*, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY. Total RNA was 10 isolated by centrifugation of cell lysates over a CsCl cushion. RNA samples were electrophoresed through 1.2% agarose-formaldehyde gels and transferred to hybridization membranes by capillary diffusion over a 12-14 hour period. The RNA was cross-linked to the membrane by exposure to UV 15 light in a Stratalinker (Stratagene, La Jolla, CA) and hybridized to a ³²P-labeled B-raf cDNA probe or G3PDH probe as a control. The human B-raf cDNA probe was cloned by PCR using complementary oligonucleotide primers after reverse transcription of total RNA. Identity of the B-raf cDNA was 20 confirmed by restriction digestion and direct DNA sequencing. RNA was quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Example 3 Antisense inhibition of cell proliferation

25 T24 cells are treated on day 0 for two hours with various concentrations of oligonucleotide and lipofectin (50 nM oligonucleotide in the presence of 2 µg/ml lipofectin; 100 nM oligonucleotide and 2.5 µg/ml lipofectin; 250 nM oligonucleotide and 6 µg/ml lipofectin or 500 nM 30 oligonucleotide and 12.5 µg/ml lipofectin). On day 1, cells are treated for a second time at desired oligonucleotide concentration for two hours. On day 2, cells are counted.

- 34 -

Example 4 Effect of ISIS 13741 on T24 Human Bladder Carcinoma Tumor Xenografts in Nude Mice

5 x 10⁶ T24 cells are implanted subcutaneously in the right inner thigh of nude mice. Oligonucleotides (ISIS 13741 and an unrelated control phosphorothioate oligonucleotide suspended in saline) are administered three times weekly beginning on day 4 after tumor cell inoculation. A saline-only control is also given. Oligonucleotides are given by intraperitoneal injection. Oligonucleotide dosage is 25 mg/kg. Tumor size is measured and tumor volume is calculated on the eleventh, fifteenth and eighteenth treatment days.

Example 5 Effect of ISIS 13741 on MDA-MB 231 Human Breast Carcinoma Tumor Xenografts in Nude Mice

5 x 10⁶ MDA-MB 231 cells are implanted subcutaneously in the right inner thigh of nude mice. Oligonucleotides (ISIS 13741 and an unrelated control phosphorothioate oligonucleotide suspended in saline) are administered once daily beginning on day 10 after tumor cell inoculation. A saline-only control is also given. Oligonucleotides are given by intravenous injection at a dosage of 2-25 mg/kg. Tumor size is measured and tumor volume is calculated on days 10, 13, 16, 20, 23 and 27 following tumor cell inoculation.

For intraperitoneal oligonucleotide administration, oligonucleotides are administered once daily beginning on day 10 after tumor cell inoculation. A saline-only control is also given. Oligonucleotides are given by intraperitoneal injection at a dosage of 2-25 mg/kg. Tumor size is measured and tumor volume is calculated on days 10, 13, 16, 20, 23 and 27 following tumor cell inoculation.

- 35 -

**Example 6 Effect of ISIS 13741 on Colo 205 Human Colon
Carcinoma Tumor Xenografts in Nude Mice**

5 x 10⁶ Colo 205 cells are implanted subcutaneously in the right inner thigh of nude mice. Oligonucleotides (ISIS 13741 and an unrelated control phosphorothioate oligonucleotide suspended in saline) are administered once per day beginning on day 5 after tumor cell inoculation. A saline-only control is also given. Oligonucleotides are given by intravenous injection. Oligonucleotide dosage is 2-25 mg/kg. Tumor size is measured and tumor volume is calculated on days 5, 8, 11, 14, 18, 22 and 25 after tumor inoculation.

**Example 7 Diagnostic Assay for raf-associated Tumors
Using Xenografts in Nude Mice**

Tumors arising from raf expression are diagnosed and distinguished from other tumors using this assay. A biopsy sample of the tumor is treated, e.g., with collagenase or trypsin or other standard methods, to dissociate the tumor mass. 5 x 10⁶ tumor cells are implanted subcutaneously in the inner thighs of two or more nude mice. Antisense oligonucleotide (e.g., ISIS 13741) suspended in saline is administered to one or more mice by intraperitoneal injection three times weekly beginning on day 4 after tumor cell inoculation. Saline only is given to a control mouse. Oligonucleotide dosage is 25 mg/kg. Tumor size is measured and tumor volume is calculated on the eleventh treatment day. Tumor volume of the oligonucleotide-treated mice is compared to that of the control mouse. The volume of raf-associated tumors in the treated mice are measurably smaller than tumors in the control mouse. Tumors arising from causes other than raf expression are not expected to respond to the oligonucleotides targeted to raf and, therefore, the tumor volumes of oligonucleotide-treated and control mice are equivalent.

- 36 -

Example 8 Detection of raf expression

Oligonucleotides are radiolabeled after synthesis by ³²P labeling at the 5' end with polynucleotide kinase. Sambrook et al., *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989, Volume 2, pg. 11.31-11.32. Radiolabeled oligonucleotides are contacted with tissue or cell samples suspected of raf expression, such as tumor biopsy samples, under conditions in which specific hybridization can occur, and the sample is washed to remove unbound oligonucleotide. Radioactivity remaining in the sample indicates bound oligonucleotide and is quantitated using a scintillation counter or other routine means.

Radiolabeled oligonucleotides of the invention are also used in autoradiography. Tissue sections are treated with radiolabeled oligonucleotide and washed as described above, then exposed to photographic emulsion according to standard autoradiography procedures. The emulsion, when developed, yields an image of silver grains over the regions expressing raf. The extent of raf expression is determined by quantitation of the silver grains.

Analogous assays for fluorescent detection of raf expression use oligonucleotides of the invention which are labeled with fluorescein or other fluorescent tags. Labeled DNA oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. β -cyanoethyldiisopropyl phosphoramidites are purchased from Applied Biosystems (Foster City, CA). Fluorescein-labeled amidites are purchased from Glen Research (Sterling VA). Incubation of oligonucleotide and biological sample is carried out as described for radiolabeled oligonucleotides except that instead of a scintillation counter, a fluorimeter or fluorescence microscope is used to detect the fluorescence which indicates raf expression.

- 37 -

Example 9 A549 xenografts

A549 cells are obtained from the American Type Culture Collection (Bethesda MD) and grown in T-75 flasks until 65-75% confluent. 5 x 10⁶ A549 cells are implanted subcutaneously in the inner thigh of nude mice. Oligonucleotides (ISIS 13741 and a scrambled raf control phosphorothioate oligonucleotide, ISIS 10353) suspended in saline are administered once daily by intravenous injection at doses ranging from 2-25 mg/kg. Resulting tumors are measured on days 9, 12, 17 and 21 and tumor volumes are calculated.

Example 10 U-87 human glioblastoma cell culture and subcutaneous xenografts into nude mice:

Ahmad et al. disclose that transfection of the human glioblastoma cell line, U-87, with vectors expressing antisense RNA to PKC α inhibits growth of the glioblastoma cells *in vitro* and *in vivo*. Ahmad et al., 1994, *Neurosurg.* 35:904-908. The U-87 human glioblastoma cell line is obtained from the ATCC (Rockville MD) and maintained in Iscove's DMEM medium supplemented with heat-inactivated 10% fetal calf serum. Nude mice are injected subcutaneously with 2 x 10⁷ cells. Mice are injected intraperitoneally with oligonucleotide at dosages of either 2 mg/kg or 20 mg/kg for 21 consecutive days beginning 7 days after xenografts were implanted. Tumor volumes are measured on days 14, 21, 24, 31 and 35 and compared to volumes obtained after treatment with saline or sense oligonucleotide control.

Example 11 Intracerebral U-87 glioblastoma xenografts in nude mice

U-87 cells are implanted in the brains of nude mice. Mice were treated via continuous intraperitoneal administration of antisense oligonucleotide (20 mg/kg), control sense oligonucleotide (20 mg/kg) or saline beginning on day 7 after

- 38 -

xenograft implantation. Survival (in days) of treated mice are compared to survival of untreated or control-treated mice.

- 39 -

What Is Claimed Is:

1. An oligonucleotide 8 to 50 nucleotides in length which is targeted to a nucleic acid encoding human B-raf and which is capable of inhibiting human B-raf expression.
2. The oligonucleotide of claim 1 which is targeted to a translation initiation site, 3' untranslated region, coding region or 5' untranslated region of mRNA encoding human B-raf.
3. The oligonucleotide of claim 1 which has at least one modified backbone linkage.
4. The oligonucleotide of claim 1 wherein at least one of the nucleotide units of the oligonucleotide is modified at the 2' position of the sugar moiety.
5. The oligonucleotide of claim 1 in a pharmaceutically acceptable carrier.
6. The oligonucleotide of claim 1 which is a chimeric oligonucleotide.
7. The oligonucleotide of claim 1 comprising SEQ ID NO: 3, 5, 7, 8, 9, 11, 13, 14, 17, 20, 21, 22, 32, 36, 33, 31, 29 or 23.
8. A method of inhibiting the expression of human B-raf comprising contacting tissues or cells which express human B-raf with an effective dose of the oligonucleotide of claim 1 whereby expression of human B-raf is inhibited.
9. The method of claim 8 wherein the oligonucleotide is in a pharmaceutically acceptable carrier.

- 40 -

10. The method of claim 8 wherein said expression of human raf is abnormal expression.
11. A method of inhibiting hyperproliferation of cells comprising contacting hyperproliferating cells with an effective dose of the oligonucleotide of claim 1, whereby hyperproliferation of cells is inhibited.
12. A method of treating or preventing an abnormal proliferative condition comprising contacting a patient suspected of having an abnormal proliferative condition, or cells, tissues or a bodily fluid of said patient, with an effective dose of the oligonucleotide of claim 1, whereby the abnormal proliferative condition is treated or prevented.
13. The method of claim 12 wherein the condition is a hyperproliferative disorder.
14. The method of claim 13 wherein the hyperproliferative disorder is cancer, restenosis, psoriasis or a disorder characterized by T-cell activation and growth.
15. The method of claim 14 wherein said cancer is a cancer of the brain or nervous system.

1/8

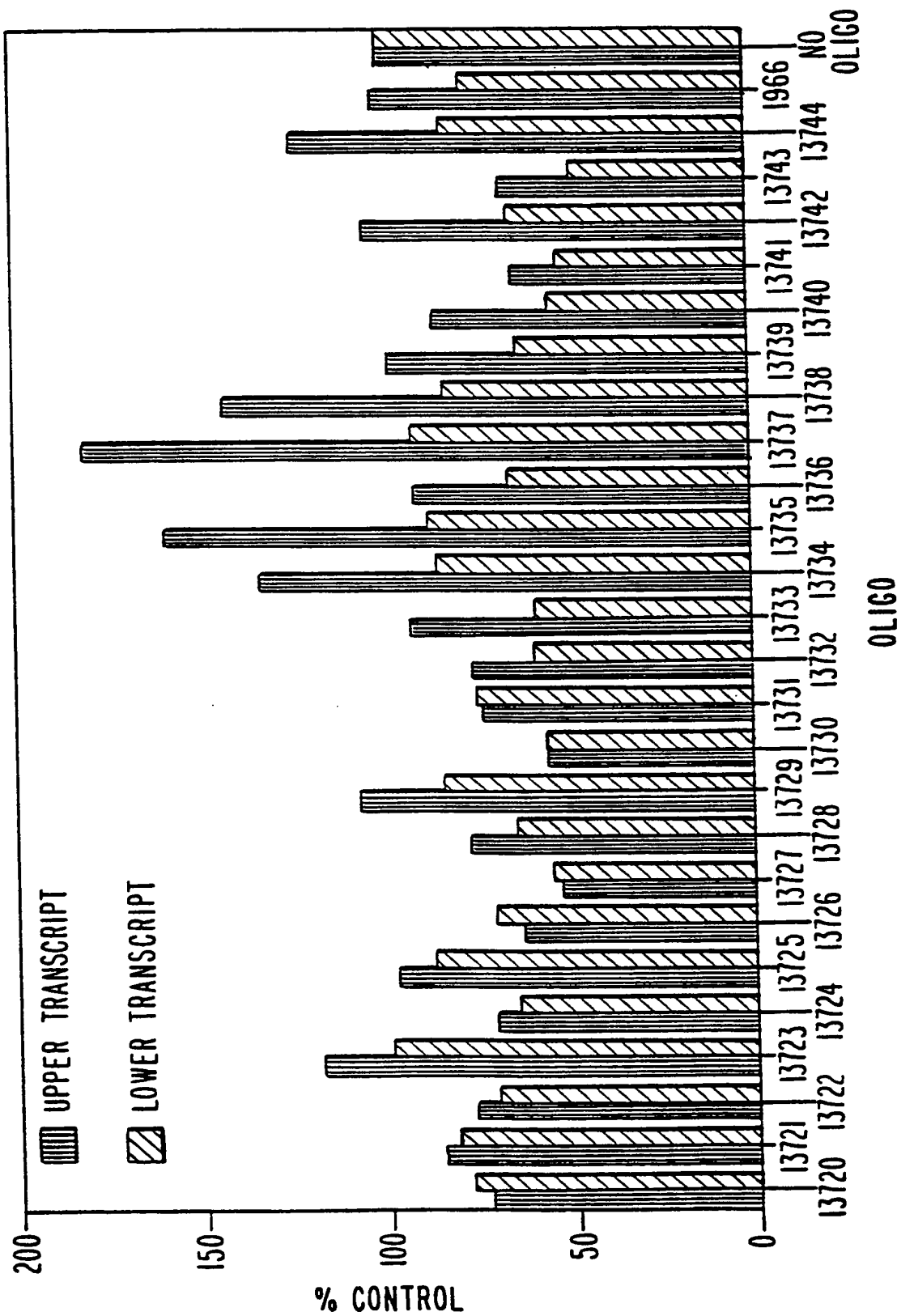
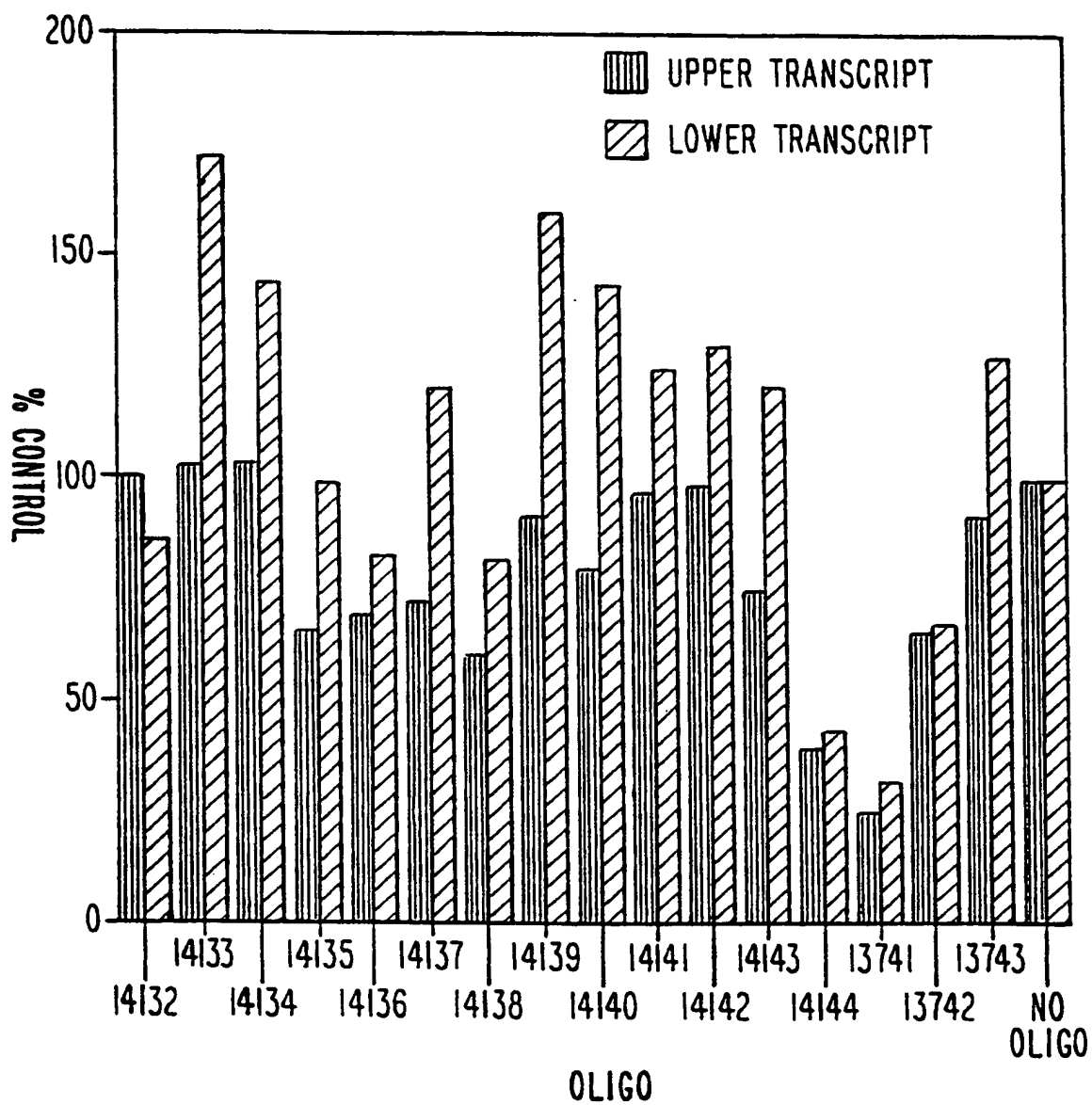


Fig. 1A

2/8

***Fig. 1B***

3/8

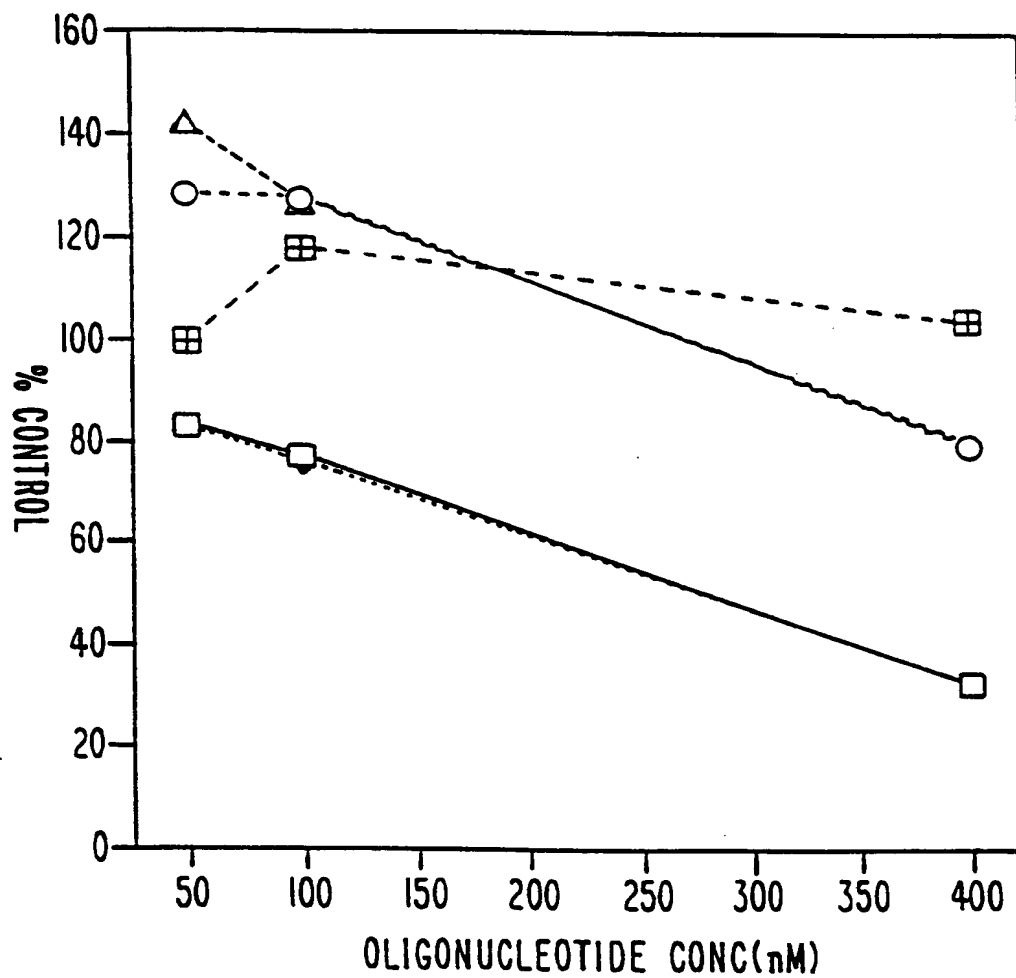


Fig. 2A

4/8

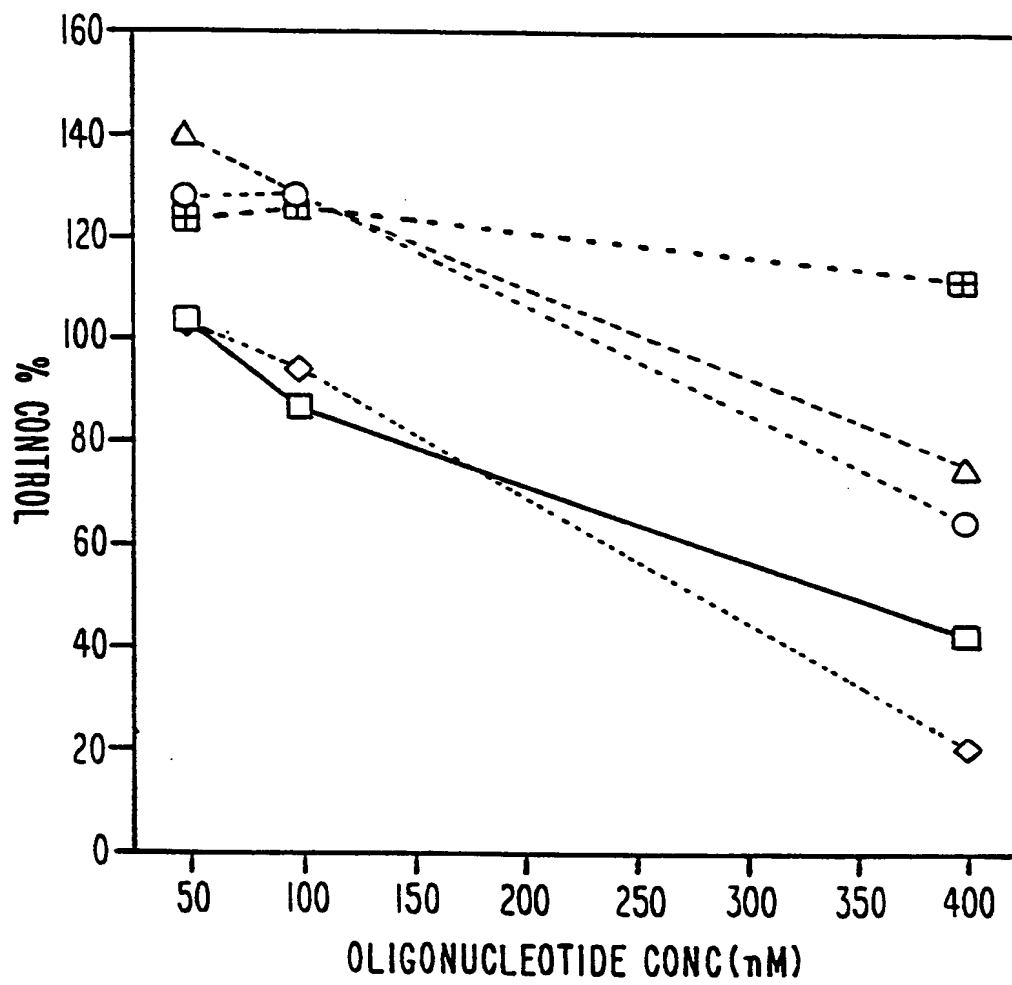
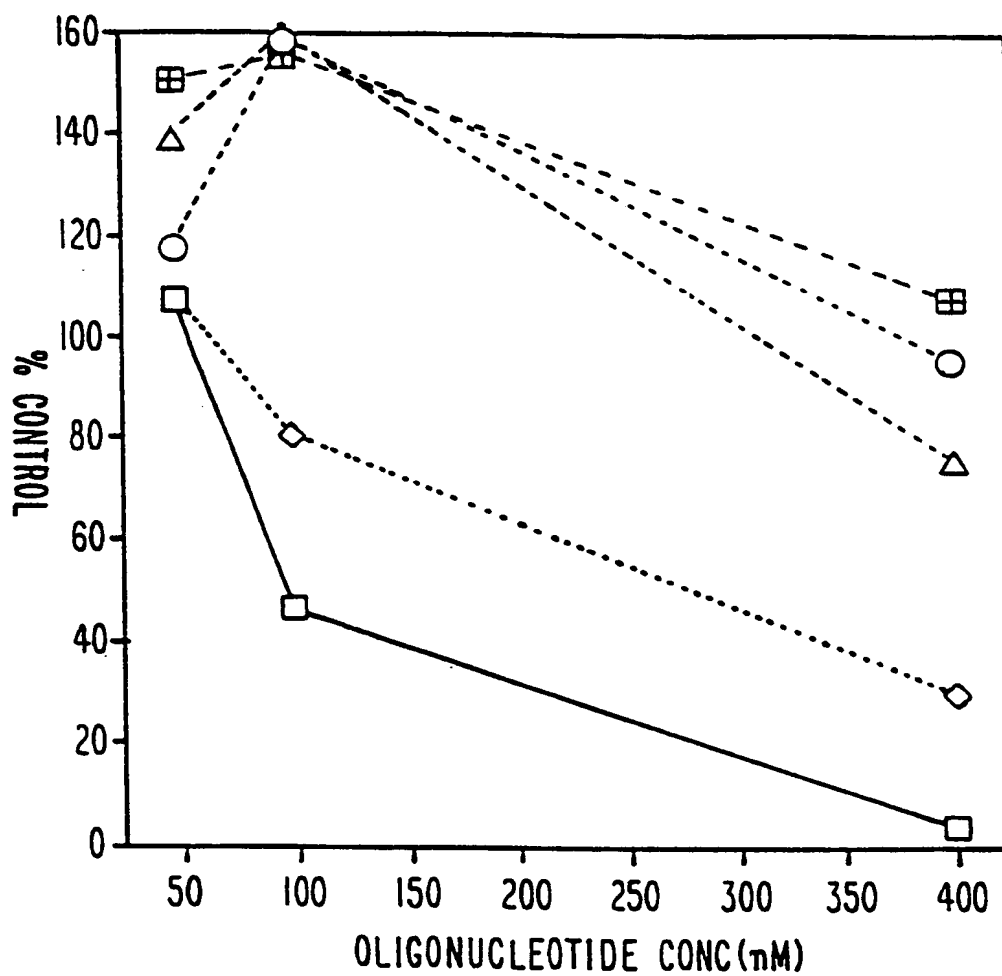
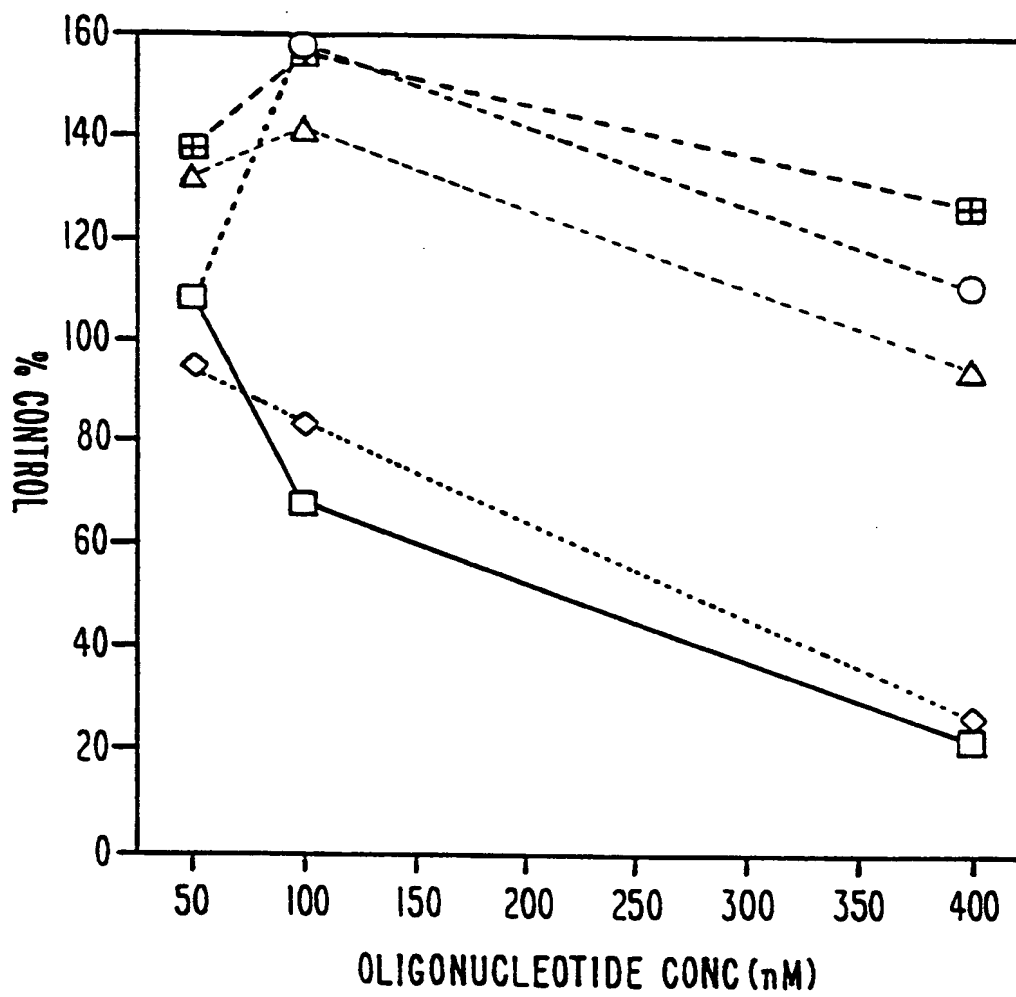


Fig. 2B

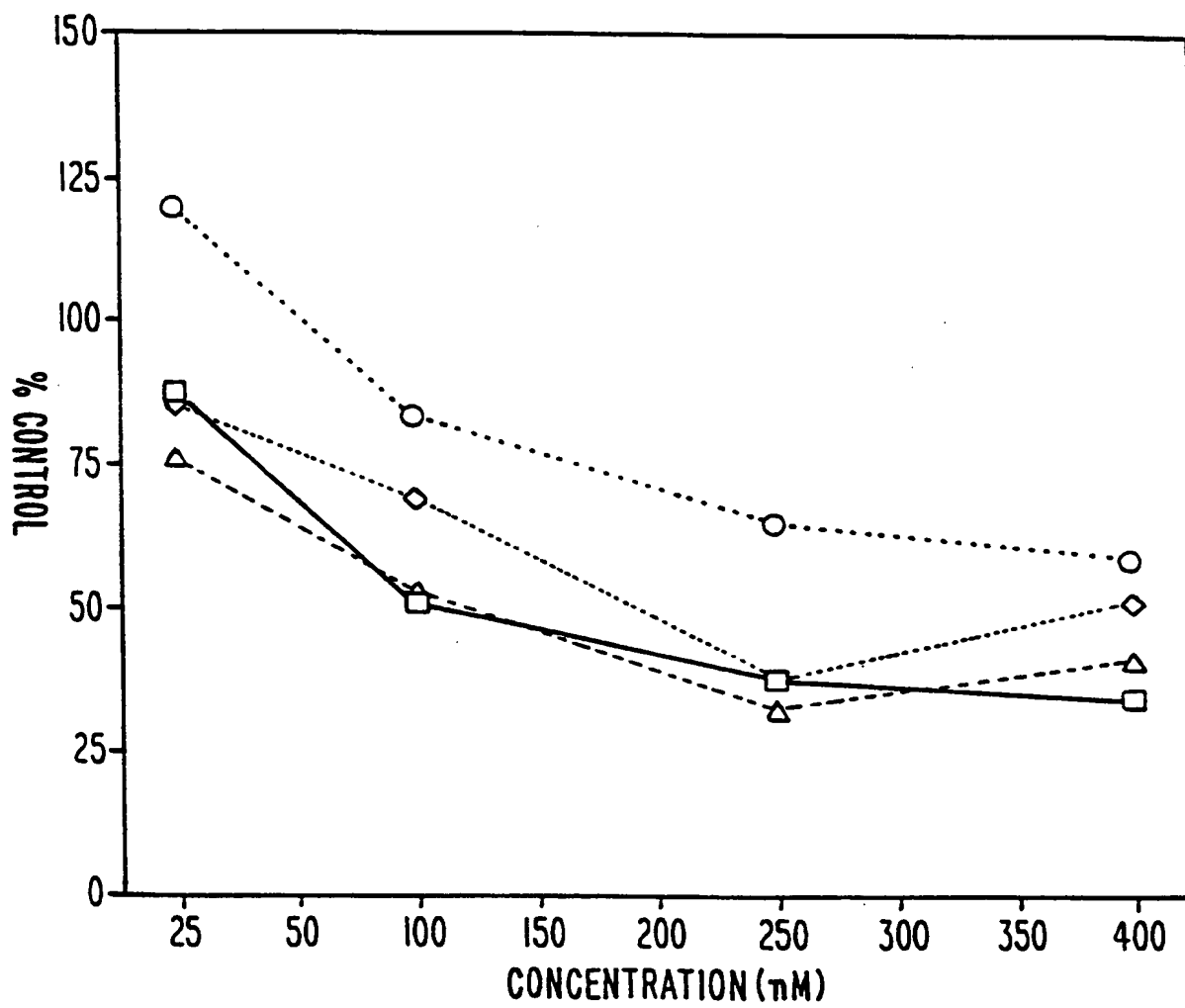
5/8

***Fig. 3A***

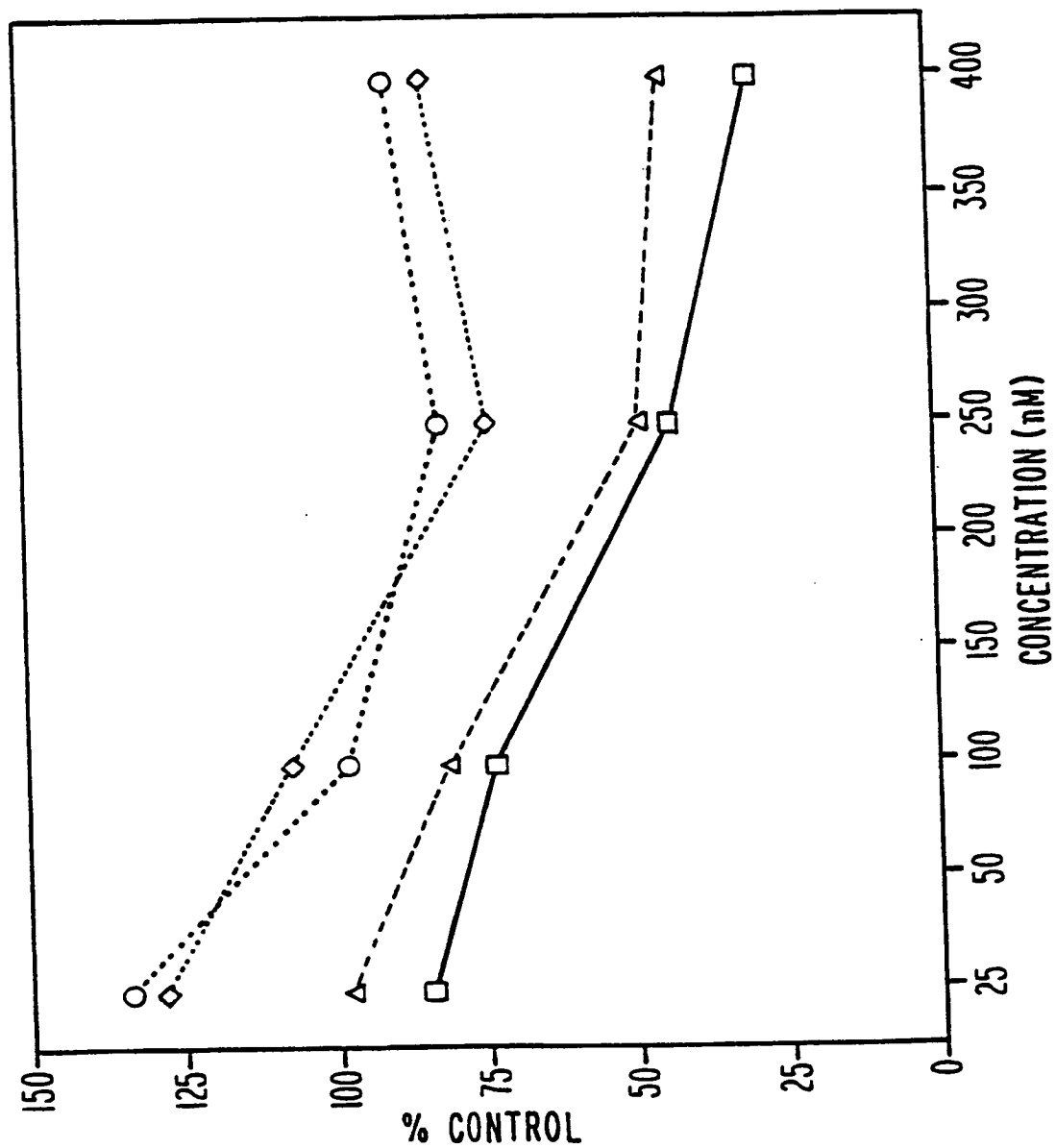
6/8

***Fig. 3B***

7/8

***Fig. 4A***

8/8

*Fig. 4B*

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Brett P. Monia

(ii) TITLE OF INVENTION: Antisense Oligonucleotide
Modulation of raf Gene Expression

(iii) NUMBER OF SEQUENCES: 42

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Jane Massey Licata, Esq.

(B) STREET: 66 East Main Street

(C) CITY: Marlton

(D) STATE: NJ

(E) COUNTRY: USA

(F) ZIP: 08053

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: Pentium

(C) OPERATING SYSTEM: Windows 95

(D) SOFTWARE: WORDPERFECT 6.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: not assigned

(B) FILING DATE: Herewith

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA 1:

(A) APPLICATION NUMBER: 08/756,806

(B) FILING DATE: November 26, 1996

(vii) PRIOR APPLICATION DATA 2:

(A) APPLICATION NUMBER: PCT/US95/07111

(B) FILING DATE: May 31, 1995

(vii) PRIOR APPLICATION DATA 3:

(A) APPLICATION NUMBER: 08/250,856

(B) FILING DATE: May 31, 1994

(vii) PRIOR APPLICATION DATA 4:

(A) APPLICATION NUMBER: 08/888,982

(B) FILING DATE: July 7, 1997

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Jane Massey Licata

(B) REGISTRATION NUMBER: 32,257

(C) REFERENCE/DOCKET NUMBER: ISPH-0312

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (609) 779-2400

(B) TELEFAX: (609) 810-1454

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATTTTGAAGG AGACGGACTG 20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TGGATTTTGA AGGAGACGGA 20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGTTAGTTAG TGAGCCAGGT 20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ATTTCTGTAA GGCTTTCACG 20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCCGTCTACC AAGTGTTTTC 20

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

AATCTCCCAA TCATCACTCG 20

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TGCTGAGGTG TAGGTGCTGT 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TGTAAGTCT GAGGTGTAGG 20

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TGTCGTGTTT TCCTGAGTAC 20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AGTTGTGGCT TTGTGGAATA 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

ATGGAGATGG TGATACAAGC 20

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGATGATTGA CTTGGCGTGT 20

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AGGTCTCTGT GGATGATTGA 20

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATTCTGATGA CTTCTGGTGC 20

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GCTGTATGGA TTTTATCTT 20

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TACAGAACAA TCCCAATGC 20

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

ATCCTCGTCC CACCATAAAA 20

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CTCTCATCTC TTTTCTTTT 20

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GTCTCTCATC TCTTTTCTTT 20

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CCGATTCAAG GAGGGTTCTG 20

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

TGGATGGGTG TTTTGGAGA 20

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CTGCCTGGAT GGGTGTTTTT 20

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GGACAGGAAA CGCACCATAT 20

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CTCATTGTGTT TCAGTGGACA 20

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

TCTCTCACTC ATTTGTTCA 20

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

ACTCTCTCAC TCATTGTTT 20

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GAACTCTCTC ACTCATTTGT 20

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

TCCTGAACTC TCTCACTCAT 20

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

TTGCTACTCT CCTGAACTCT 20

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

TTTGTTGCTA CTCTCCTGAG 20

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CTTTTGTTGC TACTCTCCTG 20

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

GCTACTCTCC TGA ACTCTCT 20

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

TTCCTTTTGT TGCTACTCTC 20

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

ATTTATTTTC CTTTGTGTC 20

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

ATATGTTTCAT TTATTTTCCT 20

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

TTTATTTTCC TTTGTGCT 20

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

TGTTCATTTA TTTTCCTTTT 20

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

ATTTAACATA TAAGCAAACA 20

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

CTGCCTGGTA CCCTGTTTTT 20

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

CTGCCTGGAA GGGTGTTTTT 20

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

CTGCCTGGTA CGGTGTTTTT

20

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2510

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

```

cgccctcccgg cccctcccc gcccgacagc ggccgctcgg gccccggctc 50
tcggttataa gatggcggcg ctgagcggtg gcggtggtgg cggcgcgagg 100
ccgggccagg ctctgttcaa cggggacatg gagccccagg ccggcgccgg 150
ccggcccgcg gcctcttcgg ctgcggaacc tgccattccg gaggaggtgt 200
ggaatatcaa acaaatgatt aagttgacac aggaacatat agaggcccta 250
ttggacaaat ttggtgggga gcataatcca ccatcaatat atctggaggc 300
ctatgaagaa tacaccagca agctagatgc actccaacaa agagaacaac 350
agttattgga atctctgggg aacggaactg atttttctgt ttctagctct 400
gcatcaatgg ataccgttac atcttcttcc tcttctagcc tttcagtgtc 450
accttcattc ctttcagttt ttcaaaatcc cacagatgtg gcacggagca 500
accccaagtc accacaaaaa cctatcggtt agtcttcct gcccacaaca 550
cagaggacag tggtagctgc aaggtgtgga gttacagtcc gagacagtct 600
aaagaaagca ctgatgatga gaggtctaata cccagagtgc tgtgctgttt 650
acagaattca ggtggagaga aagaaaccaaa ttggttggga cactgatatt 700
tcctggctta ctggagaaga attgcatgtg gaagtgttgg agaattgttc 750
acttacaaca cacaactttg tacgaaaaaac gtttttcacc ttagcatttt 800
gtgacttttg tcgaaagctg cttttccagg gtttccgctg tcaaactatg 850
ggttataaat ttcaccagcg ttgtagtaca gaagttccac tgatgtgtgt 900
taattatgac caacttgatt tgctgtttgt ctccaagttc tttgaacacc 950
acccaatacc acaggaagag gcgtccttag cagagactgc cctaaccatc 1000
ggatcatccc cttccgcacc cgcctcggac tctattgggc cccaaattct 1050
caccagtccg tctccttcaa aatccattcc aattccacag cccttccgac 1100
cagcagatga agatcatcga aatcaatttg ggcaacgaga ccgattcctc 1150
tcagctccca atgtgcatat aaacacaata gaacctgtca atattgatga 1200
cttgattaga gaccaaggat ttcgtggtga tggaggatca accacagggt 1250
tgtctgttac cccccctgcc tcattacctg gctcactaac taacgtgaaa 1300
gccttacaga aatctccagg acctcagcga gaaaggaagt catcttcac 1350
ctcagaagac aggaatcgaa tgaaaacact tggtagacgg gactcgagtg 1400
atgattggga gattcctgat gggcagatta cagtgggaca aagaattgga 1450
tctggatcat ttggaacagt ctacaaggga aagtggcatg gtgatgtggc 1500

```

```

agtgaaaatg ttgaatgtga cagcacctac acctcagcag ttacaagcct 1550
tcaaaaatga agtaggagta ctcaggaaaa cagcacatgt gaatatccta 1600
ctcttcatgg gctattccac aaagccacaa ctggctattg ttaccagtg 1650
gtgtgagggc tccagcttgt atcaccatct ccatatcatt gagaccaa 1700
ttgagatgat caaacttata gatattgcac gacagactgc acagggcatg 1750
gattacttac acgccaagtc aatcatccac agagacctca agagtaataa 1800
tatatttctt catgaagacc tcacagtaaa aataggtgat tttggtctag 1850
ctacagtgaa atctcgatgg agtgggtccc atcagtttga acagttgtct 1900
ggatccattt tgtggatggc accagaagtc atcagaatgc aagataaaaa 1950
tccatacagc tttcagtcag atgtatatgc atttgggatt gttctgtatg 2000
aattgatgac tggacagtta ccttattcaa acatcaacaa cagggaccag 2050
ataattttta tgggtgggacg aggataacctg tctccagatc tcagtaaggt 2100
acggagtaac tgtccaaaag ccatgaagag attaatggca gagtgcctca 2150
aaaagaaaag agatgagaga ccactctttc cccaaattct cgcctctatt 2200
gagctgctgg cccgctcatt gccaaaaatt caccgcagtg catcagaacc 2250
ctccttgaat cgggctgggt tccaaacaga ggattttagt ctatatgctt 2300
gtgcttctcc aaaaacaccc atccaggcag ggggatatgg tgcgtttcct 2350
gtccactgaa acaaatgagt gagagagttc aggagagtag caacaaaagg 2400
aaaataaatg aacatatgtt tgcttatatg ttaaattgaa taaaatactc 2450
tctttttttt taaggtggaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 2500
aaaaaaaacc 2510

```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13961

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 31/70, 48/00; C07H 21/00; C12N 5/08
US CL : 435/ 375; 514/44; 536/ 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 366, 375; 514/44; 536/24.3, 24.31, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,656,612 A (MONIA) 12 August 1997, column 1, line 19, column 9, line 7.	1-15
Y	US 5,563,255 A (MONIA et al.) 08 October 1996, column 1, line 21- column 14, line 48.	1-15
Y	Database BIOSIS on STN, AN 91:22888. SITHANANDAM et al. 'Complete Coding Sequence of a Human B-Raf Complementary DNA and Detection of a B-Raf Protein Kinase with Isozyme Specific Antibodies.' Oncogene. December 1990, Vol. 5, No. 12, pages 1775-1780, abstract only, see lines 1-3.	1-6, 8-15

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
G earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

25 SEPTEMBER 1998

Date of mailing of the international search report

19 OCT 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

THOMAS G. LARSON, PH.D.

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT**International application No.
PCT/US98/13961****C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MONIA et al. Antitumor Activity of a Phosphorothioate Antisense Oligodeoxynucleotide Targeted Against C-raf Kinase. Nature Medicine. June 1996, Vol. 2, No. 6, pages 668-675, see entire document.	1-6, 8-15
Y	AGRAWAL et al. Modified Oligonucleotides as Therapeutic and Diagnostic Agents. Current Opinions in Biotechnology. 1995, Vol. 6, pages 12-19, especially page 14.	4, 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13961

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN (Biosis, CAPlus, Genbank, LifeSci, INPADOC, Medline, Registry, WIPDS)

Search Terms: Raf, B-Raf, Human B-Raf, antisense, inhibit, expression, abnormal proliferation, hyperproliferation, cancer, restenosis, psoriasis, SEQ. ID. NOS: 3, 5, 7-9, 11, 13, 14, 17, 20-23, 29, 31-33, 36, and Monia, B P.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)